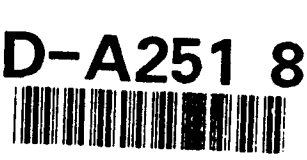

VESTAR, INC.

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Contract work for the January - April time period was focused on expanding the formulation work to include a large matrix of lipid ratios and types in order to select the most promising scale-up candidates. In addition, a variety of analytical methods were developed to aid in characterization of LEH formulations. A summary of relevant experiments is presented below:

1. Lipid sonications — Jan. 92

   Molar ranges of DSPC, DPPC, DMPC (0-100%), cholesterol (0-50%), and DMPG (0-25%) were sonicated in 5 mM phosphate/9% sucrose and 30 mM phosphate/saline to assess formation of 100 nm range liposomes. A wide variety of sizes were obtained. Both DMPG (i.e. charge) containing and phosphate/sucrose buffered liposomes exhibited more stability.

2. Surface bound hemoglobin (Hb) — Jan. 92

   A Biotin-Avidin-Ferritin labeling construct was used to attempt to discern the presence of Hb bound to the liposome exterior surface. This was unsuccessful.

3. Empty Liposomes — Jan. 92

   Liposomes were prepared without Hb to aid Somatogen in assay development.

4. Dilution Effect on Aggregation — Jan. 92

   LEH was hydrated at 115 mg/ml lipid concentration then diluted to 30 and 70 mg/ml lipid prior to homogenization to assess the effect of dilution on Hb recovery

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and aggregation. Hb recovery was low and aggregation was no different than usual.

5. Lipid sonications with rHb — Jan. 92

Formulations from item #1 which produced stable, unimodal liposomes in the 25-70 nm range were evaluated via sonication with rHb. The formulations 7:10:3 and 7:8:5 DSPC:CHOL:DMPG exhibited good profile in the 100 nm size range and fluid (not viscous) physical properties. A 50 mg/ml prep of 7:10:3 actually filtered easily through a 0.22 μm filter. This was a significant milestone.

6. Encapsulation assay. — Feb. 92

An attempt was made to separate free Hb from lipid bound Hb by utilizing centrifugation through a hydrophilic ultrafiltration membrane in a centri-prep tube. The method was unsuccessful as an encapsulation assay because of Hb-to-membrane binding.

7. Pilot scale homogenization of 7:10:3 formulation — Feb. 92

Initial production of the promising 7:10:3 candidate formulation via Gaulin homogenizer was done to identify potential areas of scale-up concern. Hydration viscosity significantly increased with mixing time. This appears to be a complex function of time, temperature, concentration, and shear rate. Resultant liposomes did not have the desired uniform small size distribution.

8. Encapsulation assay utilizing SP sephadex — Mar. 92

An encapsulation assay for Hb was developed utilizing an ion exchange resin, SP sephadex. It has been validated for precision, accuracy, and reproducibility for solutions with less than 50% free Hb.

9. Pilot scale homogenization of 7:8:5 formulation — Mar. 92

A repeat of item #7 was done but with the 7:8:5 candidate formulation. Size characteristics were not significantly improved, and the material gelled upon storage at 5° C. This and cost considerations have caused suspension of further work on this formulation in scale-up.

10. Define upper and lower limits of DSPC:CHOL:DMPG — Mar. 92

The full range of liposome forming ratios of DSPC:CHOL:DMPG were explored with rHb and evaluated via Microtrac size to establish limiting liposome and micelle forming ratios.

11. Accuracy of Tomita method for total Hb Apr. 92

Sigma Hb standards were utilized to calculate a more accurate extinction coefficient for use in the Tomita method.

12. Film vs. spray dried powder equivalence Apr. 92

Spray dried powder was produced in 7:10:3, 7:8:5:0.4 (α-tocopherol) and 7:10:3:0.4 (α-tocopherol) to assess equivalent of powders vs. films in sonication with rHb. Equivalence was demonstrated.

13. Modification of sigma method for total Hb Apr. 92

Drabkins solution (sodium bicarbonate, potassium ferricyanide, potassium cyanide, and Brij-35) coupled with OBG (octyl-β-glucopyranoside) was used to modify the Sigma method for total Hb and comparison to the Tomita method. The results were comparable, and the modified Sigma, being a one step procedure, saves time.

14. Pilot scale run with 7:10:3:0.4 Apr. 92

Alteration of hydration procedures and increased temperatures (20-25° C) were used in producing a lot of 7:10:3:0.4 via Gaulin. Liposome size characteristics were not improved significantly, but use of rHb produced via dialysis against 5 mM phosphate/9% sucrose buffer may cause problems because of high resultant metHb and/or bacteria. This will be repeated with rHb reprocessed by Somatogen with the new buffer.

15. Substitution of HSPC for DSPC and ice bath sonication Apr. 92

Empty liposomes were evaluated in the ratios of 7:8:5, 7:10:3, 6:10:4, 5:10:5, and 10:5:5 to assess the effect of substituting HSPC for DSPC. Also ice bath sonication of empty liposomes were evaluated without rHb. HSPC substitution produces liposomes similar to DSPC. Ice bath sonication results in unstable liposomes or inhomogeneous size distribution.

16. Substitution of HSPC for DSPC with rHb Apr. 92

A repeat of item #15, but no cold sonication. Presence of rHb aids in production of uniform size distributions. Bimodal empty liposome formulations such as 7:8:5 and 10:5:5 displayed this behavior.

17. Replace NRL phosphate/saline buffer using Vestar hydration method Apr. 92

NRL 10:9:1:0.2 was prepared using 5 mM phosphate/9% sucrose buffer and Vestar

hydration procedure for a future mouse toxicity evaluation. Size distribution was not significantly improved.

18. Substitute DSPG for DMPG Apr. 92

Longer chain length DSPG was substituted for DMPG in same ratios as item #15. Liposome size distribution was not as good, indicating no advantage.

19. Substitute DPPA for DMPG Apr. 92

The same change as DMPG, but with a different head group. DPPA was evaluated with the same lipid ratios as item #15. Size characteristics were similar to DMPG, but sticky character of materials made handling difficult, hence no net advantage was seen.

20. Passive rHb association with empty liposomes Apr. 92

Incubation of 67.5 mg/ml rHb with sonicated empty liposomes was done to assess association of rHb to the lipid membrane. Evaluation was done via superose 6 column. No association was seen after 3-4 hours at 25° C. However, both 4° C and 25° C showed severe binding after an overnight incubation period. Lower Hb concentrations typical of post-processing free rHb are yet to be tested.

21. Comparative toxicity in mice Apr. 92

Buffer, free rHb at 18 g/L, empty liposomes (70 mg/ml), and LEH at 18 g/L rHb from item #17 were tested for toxicity in female Balb/C mice at a dose of 0.9 g/Kg (virtually 100% topload). No toxicity was seen with buffer, rHb, or empty liposomes. The LEH caused 4 deaths in 5 animals, thus exceeding the LD_{50}. Further studies are planned to establish a baseline LD_{50}.

22. Spectrophotometric % met-Hb assay Apr. 92

Subtraction of background light scattering improved a method of spectrophotometrically determining %met-Hb. Results compare more favorably with Somatogen assay when liposome size is small and uniform than the Tomita method.

Early runs in May utilizing 7:10:3:0.4 with improved processing parameters on the Gaulin homogenizer have produced LEH which filters through 0.65 μm pore size. This is a first for LEH and is being actively pursued. Results and progress in formulation, processing, and assay development have been most encouraging during this period. Work will continue towards parameter optimization on pilot scale utilizing the Gaulin homogenizer to replicate

the sonication results seen in Jan. 92.

Sincerely,

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